EXPERIMENTAL FREEZE-DRIED MICROARTERIAL ALLOGRAFTS IN RABBITS

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ABSTRACT

Arterial allografts of 3.5 cm length were freeze-dried and placed into the femoral arteries of 20 female rabbits (16 experimental subjects and four controls). Immediate patency was 100%. Subjects were surgically explored after two months of observation. Overall patency at this time was 31% (5/16). A patency rate of 50% (5/10) was achieved with size-matched femoral grafts. However, all of the smaller diameter brachial grafts were unsuccessful. Analysis by light microscopy as well as transmission and scanning electron microscopy demonstrated intimal hyperplasia which was more prominent in nonpatent grafts. There was no evidence of a cellular immune response to the freeze-dried grafts by the host. The use of size-matched grafts and postoperative anticoagulants in future studies may improve patency rates and the potential clinical applicability of this promising microvascular technique.

INTRODUCTION

Microvascular techniques offer important alternatives for head and neck reconstruction following major tumor resections. Free flap transfers, although not yet commonplace, provide excellent tissue coverage for large defects. When this method of reconstruction is selected, the head and neck surgeon may be faced with inadequate vascular pedicle lengths or the need to anastomose vessels outside irradiated tissue beds. This procedure may require the use of interpositional vascular grafts.

In 1977, Biemer[1] used autogenous veins, which thereafter represent the gold standard against which all synthetic or preserved microvascular substitutes must be measured. There are, however, some notable limitations to their usage. For example, in certain patients, potential donor sources for venous autografts may be reduced by prior intravenous chemotherapy or the presence of obliterative vascular disease. In addition, intraoperative vessel procurement can be long, tedious and unproductive, especially in debilitated, elderly patients. Finally, there is increased morbidity associated with additional incisions and a longer procedure. It is beneficial, therefore, to have reliable prosthetic or preserved vascular grafts available for clinical use.

Laboratory studies of Broncon et al,[2] Ganske et al,[3] and Coffee,[4] investigating synthetic microvascular substitutes (primarily polytetraflouroethylene), have been inconclusive and inconsistent. On the other hand, recent reports by Chow et al[5] and Pratt et al[6] on freeze-dried vascular micrografts have been quite encouraging. Patency rates as high as 93% have been described with freeze-dried microarterial grafts in the rat model by Pratt et al.[7]

Autogenous venous micrografts have been used reliably in the rabbit by

Fujikawa and O'Brien,[8] and Dac et al.[9] Melka et al[10] achieved better success with microarterial autografts when compared to microvenous autografts in both the rabbit and the rat. Their study described superior long-term results with the microarterial grafts. In addition, Chow et al[11] had some success using freeze-dried human placental vessels as heterografts in the rabbit. All of these investigations have produced data on short-vessel segments, most commonly measuring one cm or less. If a potential microvascular substitute is to be clinically acceptable, it must be demonstrated to be reliable in longer graft segments. The present study was designed to investigate the immediate and the long-term patency in addition to the host tissue response using 3.5 cm freeze-dried microarterial interposition allografts in the rabbit model.

MATERIALS AND METHODS

Femoral and brachial arteries were harvested in 3.5 to 4.0 cm lengths from New Zealand white female rabbits. All branches from each segment were suture ligated and incised. These donor arteries were lyophilized slowly over a 24 hour period in the Virtis Bench Top 3 Model Freeze Dryer (The Virtis Co., Inc., Gardiner, NY) and stored in 15 ml vacuum sealed vials at room temperature. Prepared vessels were rehydrated in normal saline (50 ml) for 20 minutes before grafting.

The subjects in the study were New Zealand white female rabbits weighing between 2.6 and 5.8 kg. General anesthesia was induced with IM Ketamine (50 mg/kg) and Xylazine HCl (20 mg/kg), an animal tranquilizer. Anesthesia was maintained with oxygen, and halothane was delivered by mask. The groin was shaved, and a surgical depilatory was applied to the area. Using aseptic technique, the femoral vessels were exposed on the right side through a

longitudinal incision in the medial thigh. The femoral artery (2.2 to 2.3 mm internal diameter) was topically irrigated with 1% lidocaine to relieve spasm.

There were two experimental groups, one received the brachial grafts and the other received the femoral grafts. In both experimental groups, microvascular clamps were placed and a 2.0 cm segment of the femoral artery was excised. This left a 3.5 cm defect secondary to vessel retraction from elastic recoil. The cut ends of the arteries were then flushed with heparinized saline (330 units/ml), and the adventitia was trimmed. The previously prepared freeze-dried brachial (1.8 to 2.0 mm internal diameter) and femoral (2.2 to 2.3 mm internal diameter) arteries were used as interposition grafts (Fig. 1). End-to-end anastomoses were performed with 10-0 Ethilon suture on a BV75 needle using the Carl Zeiss OPMI-6SD microscope. Eight interrupted sutures were used to perform each anastomosis with the freeze-dried graft. Average ischemia time was 50 minutes in experimental subjects (range 35 to 90 minutes).

In the control group, a 3.5 cm segment was removed and used as an autograft. As a consequence of elastic retraction of the vessel walls, a 4.5 cm defect was created. Therefore, each control subject had a 3.5 cm graft placed into a 4.5 cm defect, whereas each experimental subject had a 3.5 cm graft placed into a 3.5 cm defect. Twelve sutures were required in the control anastomoses in order to overcome the high tension generated by vessel retraction. Average ischemia time was 70 minutes in control subjects (range 50 to 90 minutes).

Microvascular clamps were removed and immediate patency at each anastomosis was assessed by standard techniques. These included the flicker test, strip test and the presence of expansile pulsation. The skin incision was closed with running 4-0 Vicryl sutures.

The groin was surgically explored at two months, and the femoral vessels were examined. Long-term patency was determined at this time by incising the femoral artery distal to both anastomoses and observing for brisk blood flow. Perfusion fixation of the grafts was performed with glutaraldehyde solution using the technique of Derman and Schenk.[12]

Grafts were harvested, analyzed and each specimen was sectioned longitudinally. One half was prepared for transmission electron microscopy evaluation and specimens were osmicated, dehydrated, embedded in Medcast and stained with uranylacetate and lead citrate. Segments were stained with toluidine blue and examined under a light microscope. The other half was evaluated by scanning electron microscope and specimens were osmicated, dehydrated, critical point dried and gold coated.

There were 21 subjects in the sample group. These subjects comprised one control group and two experimental groups. Of the experimental groups, one contained six brachial graft subjects and the other contained 11 femoral graft subjects. There were four subjects in the control group. One experimental subject from the femoral group died of anesthetic complications following successful graft interposition and was excluded from the study. This left a total of 16 experimental subjects and four control subjects for evaluation.

RESULTS

All grafts were patent immediately following the surgical procedure. At two months, the overall patency rate was 31% (5/16) for the experimental group and 0% (0/4) for the control group. Although none of the smaller diameter brachial grafts (0/6) were patent at the time of exploration, 50% (5/10) of the size-matched femoral grafts were patent and exhibited brisk flow when the distal femoral artery was incised. The nonpatent grafts were surrounded by

extensive fibrosis. Seven of the nonpatent grafts were flat and attenuated suggesting early failure; however, three grafts were round and contained firm, well organized thrombus formations. There was one occlusive aneurysm in a nonpatent graft. The five patent grafts were surrounded with minimal fibrosis.

Descriptions of microscopy data will be based on the presence or absence of patency. Light microscope examination of the patent grafts revealed minimal intimal hyperplasia in the native femoral artery which was continuous with a neointimal lining in the freeze-dried graft. There was no evidence of lymphocytic infiltration in the wall of any graft. Increased intimal hyperplasia was noted in nonpatent grafts.

Examination of patent grafts by electron microscopy revealed a single layer neointima with a mature tunica media (Fig. 2). The endothelium of the grafts took on the appearance of the native vessel (Fig. 3), except that it had an undulating surface (Fig. 4). The elastic membrane was highly organized. The tunica media contained an abundance of active cells (fibroblasts and myoblasts) with prominent rough endoplasmic reticulum. No lymphocytes were discovered in the wall of any graft. Endothelial pseudopodia from the native femoral artery projected over the graft surface to form a neointimal lining (Fig. 5). These findings correlated well with data obtained by light microscopy.

The data were analyzed by applying two statistical tests. Due to the error introduced by creating a larger arterial defect in the control rabbits, this group was deleted from statistical analysis. Patency rates from the two experimental groups were compared using the Fisher Exact Probability Test. Differences between the results obtained in the brachial and femoral groups were not statistically significant. However, a definite trend was noted

($p = .06$).

The data from the femoral group were then analyzed using the binomial test which compares experimental results to an empirical norm. As previously stated, the autogenous vein graft is the standard against which all vascular substitutes must be compared. Fujikawa and O'Brien achieved 84% patency (16/19) with 40 mm interpositional femoral vein autografts placed into rabbit femoral arterial defects. As their's is the only published study of longer microvenous autografts in rabbits, their patency rate was used as an empirical norm. There was insufficient published data to establish a theoretical norm. Using this value (84% patency), the null hypothesis, which stated there was no statistical difference between obtained results and the established empirical norm, was rejected ($p < .01$, one tailed). Stated otherwise, a statistically significant difference was established between the freeze-dried femoral artery allografts used in this study and microvenous femoral allografts which produced higher patency rates in Fujikawa's study using the same animal model.

COMMENT

The results of this study revealed several potential problems with the use of freeze-dried microarterial grafts. The patency rates were unsatisfactory, and this observation will be analyzed in depth. The intimal hyperplasia, more prevalent in the nonpatent grafts by microscopy, is well described by Berguer et al.[13] This is the expected tissue response to acute vascular injury and it is enhanced by low blood flow states induced by thrombogenesis. There was no evidence of an observable cellular immune response according to the microscopy studies; micrographs indicated no lymphocytic infiltration in graft walls. This finding could represent a very beneficial characteristic of freeze-dried vascular grafts but it bears further investigation. Each of these points will now be discussed in detail.

Preserved microvascular substitutes would be beneficial to the head and neck surgeon as long as their patency rates approach those of microvenous interposition autografts (80 to 90%), as reported by Biemer[1] and Fujikawa and O'Brien.[8] Surgical technique, low blood flow states and thrombogenicity of the graft material affects the potential for thrombosis. In small diameter vessels (2 mm and below), Barnes[14] states that a minute change in internal diameter can substantially reduce blood flow rates and lead to occlusion. Normal endothelium is resistent to thrombosis; however, and Ackland[15] notes that endothelium interrupted at a suture anastomosis enhances thrombosis and potential graft failure. This occurs when platelets are activated and chemotactic factors are released thereby initiating thrombogenesis. In order to prevent vessel thrombosis, it has become a common practice for clinical microvascular surgeons to use postoperative antiplatelet and anticoagulant therapy. Kolar et al[16] have reported on improved patency in coagulated rabbits. In 1974, Kogma et al[17] revealed that the mechanism for the blood coagulation in the rabbit is quite similar to that in the human, and it seems apparent that the success of investigations with freeze-dried micrografts in rabbits may be improved by incorporating antiplatelet and anticoagulant therapies. This was not done in our study.

Surgical technique, another important variable to consider when evaluating patency rates, was relatively constant. Each procedure was performed by two experienced microvascular surgeons working as a team. The only procedural differences were related to the graft material. There was a notable size mismatch between the native femoral arteries and the freeze-dried brachial grafts which were not readily distensible. The placement of these smaller, nondistensible grafts would be expected to reduce blood flow, increase

turbulence at each anastomosis and thereby increase thrombogenic potential. Although statistical analysis suggests no difference between the brachial and femoral grafts, the clinical significance of the difference in patency is apparent. Since technique was relatively constant, the only variable between these groups was the internal diameter of the vascular grafts. It is logical, therefore, to consider this an important factor in explaining the vastly different patency rates.

The control procedures, which were all unsuccessful, contained one important difference in comparison with experimental procedures. Since the control autografts were placed into defects enlarged by the normal retraction of vessel ends, tension across each anastomosis was considerably increased. In the experimental procedures, a 2.0 cm segment of femoral artery was excised, leaving a 3.5 cm defect influenced by intrinsic elastic retraction, into which a 3.5 cm freeze-dried graft of identical length was interposed. Thus tension was minimized in the experimental subjects. This was not so in the control subjects, which had a 3.5 cm graft placed into a 4.5 cm defect. The increased tension necessitated the use of more sutures to complete each control anastomosis. These factors selectively increased thrombogenicity and created bias in the control group. This represented a weakness in the study. A better control design would have utilized grafts taken from the contralateral femoral arteries. These autografts could then have been placed into defects which were identical to those created in the experimental subjects. Even though this would have involved an additional groin incision in control subjects, procedural bias would have been minimzed.

Bergner et al[13] have reported that intimal hyperplasia is a common cause of failure in microvascular grafts. Low blood flow rates are associated

with more extensive intimal hyperplasia which can cause occlusion. Many of the nonpatent grafts in this study showed prominent intimal hyperplasia as expected. The patent grafts had no evidence of this histopathology but rather had smooth, single-layer intimal linings. These data support similar findings recently reported by Pratt et al[7] with freeze-dried microarterial grafts.

Review of the micrographs showed no observable evidence of lymphocytic infiltration in the freeze-dried graft walls. As was reported by Pratt et al,[7] this finding suggests the absence of a similar immune response to the freeze-dried microarterial grafts by the host. A stronger statement about the lack of immunogenicity could be made by analyzing and quantifying the host immune response to these grafts with immunohistochemical studies in future investigations.

There was a significant difference between results obtained with freeze-dried microarterial grafts and the results expected using an empirical norm (microvenous autografts). In evaluating these data, it is important to consider all of the limitations mentioned in this discussion. The use of size matched arterial interposition grafts, as well as postoperative antiplatelet and anticoagulant therapies in future studies may improve patency rates. The introduction of an improved control design would also assist with data interpretation. Immunohistochemical studies to further document the absence of a cellular immune response to the freeze-dried grafts would be beneficial.

With the limitations of the present study notwithstanding, this new microvascular technique shows promise. Eventually, it is anticipated that freeze-dried placental or donor vessels could be used in clinical trials if future preliminary laboratory studies in rabbits and larger animals are successful.

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LEGENDS

Figure 1. Freeze-dried microarterial interposition graft placed into the right femoral artery through a longitudinal groin incision.

Figure 2. Transmission electron micrograph of a freeze-dried artery harvested at two months showing a patent lumen (L), a single layer endothelium, viable fibroblasts (F) and mature collagen (C). (original magnification x 1,900)

Figure 3. Scanning electron micrograph (SEM) showing smooth proximal endothelium similar to native artery. Patent lumen contains RBC clot (arrow) not attached to vessel wall - artifact of fixation. (original magnification x 20)

Figure 4. SEM appearance of undulating endothelial surface (arrow) in a freeze-dried arterial graft. (original magnification x 200)

Figure 5. SEM showing endothelial pseudopodia from the proximal native artery projecting onto the inner surface of the freeze-dried graft. (original magnification x 5,000)